

# The Two CcdA Proteins of *Bacillus anthracis* Differentially Affect Virulence Gene Expression and Sporulation

Hesong Han, Adam C. Wilson

Department of Biology, Georgia State University, Atlanta, Georgia, USA

The cytochrome *c* maturation system influences the expression of virulence factors in *Bacillus anthracis*. *B. anthracis* carries two copies of the *ccdA* gene, encoding predicted thiol-disulfide oxidoreductases that contribute to cytochrome *c* maturation, while the closely related organism *Bacillus subtilis* carries only one copy of *ccdA*. To investigate the roles of the two *ccdA* gene copies in *B. anthracis*, strains were constructed without each *ccdA* gene, and one strain was constructed without both copies simultaneously. Loss of both *ccdA* genes results in a reduction of cytochrome *c* production, an increase in virulence factor expression, and a reduction in sporulation efficiency. Complementation and expression analyses indicate that *ccdA2* encodes the primary CcdA in *B. anthracis*, active in all three pathways. While CcdA1 retains activity in cytochrome *c* maturation and virulence control, it has completely lost its activity in the sporulation pathway. In support of this finding, expression of *ccdA1* is strongly reduced when cells are grown under sporulation-inducing conditions. When the activities of CcdA1 and CcdA2 were analyzed in *B. subtilis*, neither protein retained activity in cytochrome *c* maturation, but CcdA2 could still function in sporulation. These observations reveal the complexities of thiol-disulfide oxidoreductase function in pathways relevant to virulence and physiology.

*Bacillus anthracis* is a Gram-positive, endospore-forming bacterium that is the etiological agent of anthrax. Several virulence factors, including the anthrax toxin and capsule, contribute to virulence in the mammalian host (1). Expression of both the toxin and capsule genes is dependent upon the master virulence regulator AtxA. Loss of *atxA* strongly decreases transcription of toxin and capsule genes (2, 3) and renders *B. anthracis* avirulent in animal models of infection (4). The direct interaction between AtxA and the promoters of its target genes has not been demonstrated, and the mechanism by which AtxA regulates its target genes remains unclear. AtxA activity is regulated posttranscriptionally by alternate phosphorylation and dephosphorylation (5), the global regulator CodY (6), and multimerization in response to growth conditions (7). *atxA* transcription is directly regulated by the transition state regulator AbrB, which connects virulence regulation to the sporulation pathway (8). *atxA* transcription is also indirectly regulated in response to glucose through the catabolite repressor protein CcpA (9).

We recently reported that loss of the two small *c*-type cytochromes of *B. anthracis*, *c*<sub>550</sub> and *c*<sub>551</sub>, leads to increased transcription of *atxA* and increased production of AtxA-regulated virulence factors (10). These observations led us to further investigate the roles of *c*-type cytochromes and cytochrome *c* maturation pathways in *B. anthracis* physiology and virulence. The *c*-type cytochromes are distinct from other cytochromes due to their covalently attached heme (11). Maturation *c*-type cytochromes is a complex, multistep process (12). While little is known about cytochrome production and activity in *B. anthracis*, our understanding is informed by findings in the closely related organism, *Bacillus subtilis*. *B. subtilis* carries a system II cytochrome *c* maturation system that is similar to those found in Gram-positive bacteria, cyanobacteria, the beta-, delta-, and epsilonproteobacteria, and chloroplasts (12, 13). The *c*-type cytochrome is initially produced as an apoprotein in the cytoplasm, missing its required cofactor, heme. The apocytochrome *c* is secreted across the membrane by the Sec translocation system. At the membrane, the cytochrome *c* maturation protein ResA reduces cysteine residues in the heme

binding pocket of apocytochrome (14), while ResB and ResC transport and covalently attach heme to the reduced apocytochrome to generate the final, active form of cytochrome *c* (15, 16).

The thiol-disulfide oxidoreductase protein CcdA is also required for cytochrome *c* maturation in *B. subtilis*. CcdA is an integral membrane protein with six transmembrane (TM) domains with conserved cysteine residues in TM1 and TM4 (17, 18). The protein is homologous to a portion of *Escherichia coli* DsbD (18, 19). CcdA was first identified in *B. subtilis* during a screen for mutations that result in decreased production of *c*-type cytochromes (18). Loss of CcdA also results in disruption of late events in spore formation, indicating multiple extracellular functions of CcdA (20, 21). Subsequent work has shown that CcdA accepts electrons from thioredoxin in the cytoplasm and transfers the electrons to ResA at the cell surface (22). Reduced ResA can then reduce a disulfide linkage on apocytochrome *c*, facilitating covalent attachment of heme and production of functional *c*-type cytochromes (14, 23). The phenotypes associated with loss of CcdA can be suppressed by mutations in BdbC and BdbD, two other protein thiol-disulfide oxidoreductases that are involved in an oxidative pathway, because the disulfide bond in apocytochrome *c* that is reduced by ResA is no longer formed (14, 24). In addition to its interactions with ResA, CcdA reduces StoA, which contributes to spore formation and may reduce YneN, which has no known function (12, 25).

In the *B. anthracis* genome, there are two genes predicted to encode CcdA-like proteins (26), a feature found in members of

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Address correspondence to Adam C. Wilson, acwilson@gsu.edu.

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the pathogenic *Bacillus cereus* group but not found in many other groups of bacilli (27). It is unclear why *B. anthracis* carries two copies of *ccdA* when *B. subtilis* can carry out both efficient sporulation and *c*-type cytochrome production with a single copy of *ccdA*. In this report, we show that the duplicate CcdA proteins have dissimilar activities in the cytochrome *c* maturation and spore formation pathways and that these differences are reflected in their gene expression profiles. Further, the activities of the two *B. anthracis* CcdA proteins are altered when expressed in *B. subtilis* and are distinct from the activity of endogenous *B. subtilis* CcdA.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *B. anthracis* strain 34F2(pXO1<sup>+</sup> pXO2<sup>−</sup>) and its derivatives were routinely grown in LB or brain heart infusion (BHI) broth supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol, 7.5 µg/ml; erythromycin, 5 µg/ml; lincomycin, 25 µg/ml; and kanamycin, 7.5 µg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 µg/ml) was added to LB agar to monitor β-galactosidase activity as necessary. Competent cells of *B. anthracis* were prepared by following the method of Koehler et al. (28), and electroporation was performed using the Bio-Rad Gene Pulser according to the instructions of the supplier.

*B. subtilis* strain JH642 and its derivatives were routinely grown in LB or nutrient sporulation medium phosphate (NSMP) (29) supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol, 5 µg/ml; erythromycin, 5 µg/ml; lincomycin, 25 µg/ml; and kanamycin, 5 µg/ml. *B. subtilis* transformation was performed as previously described (30).

*Escherichia coli* TG1, C600, and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed in chemically competent cells as previously described (31). Transformants were selected on LB agar supplemented with ampicillin (100 µg/ml), chloramphenicol (7.5 µg/ml), or kanamycin (30 µg/ml).

**Plasmid and strain construction.** Strains and plasmids are listed in Table 1. Oligonucleotide primers are listed in Table S1 in the supplemental material. Markerless gene deletions in *B. anthracis* and *B. subtilis* were generated through a modification of the technique of Janes and Stibitz (32), as previously described (10), with the exception that plasmid pSS4332 (33) was used in place of plasmid pBKJ223 (32). The retention of plasmid pXO1 in *B. anthracis* strains was confirmed by PCR using primers atxAU5' Bam and atxAD3' Pst.

The expression plasmid pAW285 was created by PCR amplification of JH642 genomic DNA to produce a DNA fragment containing the xylose repressor, *xylR*, and the XylR-regulated *xylA* promoter of *B. subtilis* by using primers *xyl5'*Eco and *xyl3'*Bam. The fragment was then cloned into plasmid pHP13 (34) at the EcoRI and BamHI sites. The resulting plasmid allows subcloning of genes downstream of the XylR-repressed *xylA* promoter, thereby placing their expression under a strong constitutive promoter in *B. anthracis* that can be further induced by xylose.

**β-Galactosidase assays.** *B. anthracis* strains harboring gene promoter fusions on the replicative vector pTCV-lac (35) were grown at 37°C in LB supplemented with the appropriate antibiotics. β-Galactosidase activity was assayed as described previously, and specific activity was expressed in Miller units (36, 37).

**Quantitative RT-PCR.** RNA was extracted from *B. anthracis* using the UltraClean microbial RNA isolation kit (MoBio, Carlsbad, CA). RNA was treated with Turbo DNase (Life Technologies, Carlsbad, CA) and quantified using an Eppendorf BioSpectrophotometer. Quantitative, real-time reverse transcription (RT)-PCR was performed using the Power SYBR RNA-to-Ct 1-step kit (Life Technologies) on a Prism 7500 Fast real-time PCR system (Life Technologies). Data are presented as threshold cycle (ΔΔC<sub>T</sub>) values calculated from the results of at least three independent experiments.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source
<i>B. anthracis</i> strains		
34F2	pXO1 <sup>+</sup> pXO2 <sup>−</sup>	Laboratory stock
AW-A027	Markerless deletion of <i>ccdA1</i>	This study
AW-A028	Markerless deletion of <i>ccdA2</i>	This study
AW-A029	Markerless deletion of <i>ccdA1</i> and <i>ccdA2</i>	This study
34F2ΔresB	Markerless deletion of <i>resB</i>	10
<i>B. subtilis</i> strains		
JH642	<i>pheA1 trpC2</i>	Laboratory stock
AW-S002	Markerless deletion of <i>ccdA</i>	This study
Plasmids		
pORI-Cm-I-SceI	pORI-Cm vector with I-SceI recognition site, Cm <sup>r</sup>	
pSS4332	I-SceI expression plasmid, Kan <sup>r</sup>	33
pTCVlac- <i>pagA</i>	<i>pagA-lacZ</i> transcriptional fusion in pTCV-lac, Kan <sup>r</sup>	5
pTCVlac- <i>atxA12</i>	<i>atxA-lacZ</i> transcriptional fusion in pTCV-lac, Kan <sup>r</sup>	41
pTCV-lac	Promoterless vector for transcriptional <i>lacZ</i> fusion, Kan <sup>r</sup>	35
pAW184	Regions flanking BAS1647 in pORI-Cm-I-SceI, Cm <sup>r</sup>	This study
pAW186	Regions flanking BAS3455 in pORI-Cm-I-SceI, Cm <sup>r</sup>	This study
pHP13	Cloning/shuttle plasmid, Cm <sup>r</sup>	34
pAW285	Xylose-inducible expression plasmid, Cm <sup>r</sup>	This study
pAW304	<i>ccdA1</i> expression vector, Cm <sup>r</sup>	This study
pAW305	<i>ccdA2</i> expression vector, Cm <sup>r</sup>	This study
pAW306	<i>B. subtilis ccdA</i> expression vector, Cm <sup>r</sup>	This study
pAW350	<i>ccdA2-lacZ</i> transcriptional fusion in pTCV-lac, Kan <sup>r</sup>	This study
pAW355	<i>ccdA1-lacZ</i> transcriptional fusion in pTCV-lac, Kan <sup>r</sup>	This study
pAW360	Regions flanking <i>B. subtilis ccdA</i> in pORI-Cm-I-SceI, Cm <sup>r</sup>	This study

<sup>a</sup> Cm<sup>r</sup>, chloramphenicol resistance; Kan<sup>r</sup>, kanamycin resistance.

**TMPD oxidase staining.** The presence of an active *c*-type cytochrome was verified by a colorimetric assay using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as an artificial electron donor that can be oxidized by cytochrome *caa*<sub>3</sub>. TMPD oxidase staining was performed on *B. anthracis* or *B. subtilis* grown on NSMP as previously described (10, 16).

**Sporulation analysis.** Sporulation assays were performed using strains grown in NSMP or Schaeffer's sporulation medium (SM) (38), containing chloramphenicol if necessary, at 37°C for 48 h. Sporulation was initially monitored by visualization of cells on a Zeiss Axio Imager microscope and enumeration of spores and vegetative cells from multiple independent fields. Sporulation efficiency was assayed by chloroform treatment and enumeration of spores and vegetative cells as previously described (39). Sporulation efficiency is presented as percentage of spores relative to total viable cells.

**Operon and transcription analysis.** Operon organization was determined by conventional RT-PCR by using 34F2 RNA extracted using the UltraClean microbial RNA isolation kit. Following extraction, RNA was treated with Turbo DNase. cDNA was synthesized using SuperScript III reverse transcriptase and random hexamers (Life Technologies). Sets of primers flanking the intergenic regions for each gene pair were used to amplify cDNA. Amplicons were separated by electrophoresis on 1% aga-

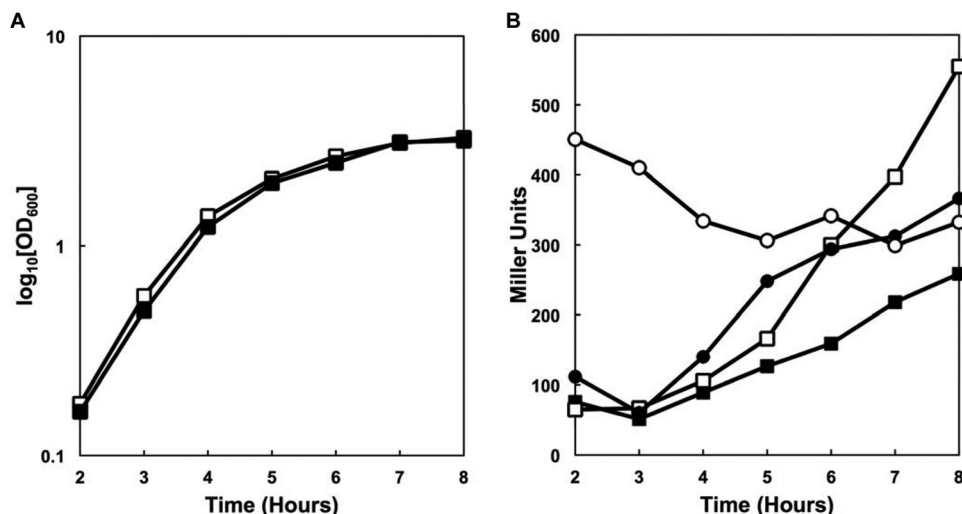


FIG 1 Growth and virulence gene expression in the *ccdA1 ccdA2* mutant strain. (A) Cell growth of parental and mutant strains grown in LB at 37°C. ■, 34F2; □, AW-A029 (*ccdA1 ccdA2*). (B)  $\beta$ -Galactosidase activity in *pagA* and *atxA* reporter strains grown in LB supplemented with kanamycin at 37°C. ■, 34F2 *pagA-lacZ*; ●, 34F2 *atxA-lacZ*; □, AW-A029 *pagA-lacZ*; ○, AW-A029 *atxA-lacZ*.

rose gels, stained by ethidium bromide, and visualized on a UVP gel documentation system.

For gene expression analysis, the intergenic regions upstream of the first linked gene of each operon were amplified using primers indicated in Table S1 in the supplemental material. The PCR fragments were then cloned into pTCV-lac (35), and sequences were confirmed by DNA sequencing. Transcriptional activities of the promoter fragments were then tested under a variety of conditions using  $\beta$ -galactosidase analysis.

## RESULTS

**Identification and description of *ccdA1* and *ccdA2* loci.** Disruption of the cytochrome *c* maturation pathway in *B. anthracis* causes increased transcription of virulence genes, the result of the loss of the two small *c*-type cytochromes *c*<sub>550</sub> and *c*<sub>551</sub> (10). In previous transposon mutagenesis screens for mutants that cause increased transcription of virulence genes, the *B. anthracis* orthologs of most nonessential genes known to be required for cytochrome *c* maturation in the model organism *B. subtilis* were disrupted (10, 16). A gene important to cytochrome *c* production in *B. subtilis* is *ccdA*, which encodes a protein thiol-disulfide oxidoreductase that contributes to covalent attachment of heme to apocytochrome *c* at the cell surface (18, 21, 24). *ccdA* is also involved in spore formation in *B. subtilis* (20). A *ccdA* disruption was not isolated in a screen for mutants with increased transcription of virulence genes that isolated mutants in most other cytochrome *c* maturation genes (10). Examination of the *B. anthracis* genome reveals the presence of two *ccdA*-like genes. BAS1647 (GenBank accession no. AAT53964.1) encodes a protein with 65% amino acid similarity to *B. subtilis ccdA* (GenBank accession no. CAB13677.1). BAS3455 (GenBank accession no. AAT55761.1) encodes a protein with 68% amino acid similarity to *B. subtilis ccdA*. BAS1647 and BAS3455 share 63% amino acid similarity. We will refer to BAS1647 as *ccdA1* and BAS3455 as *ccdA2*. The lack of *ccdA* disruption mutants in our previous screen may be due to the existence of two complementary copies of *ccdA*.

The genomic context of both *ccdA1* and *ccdA2* is different than that of *B. subtilis ccdA*. *B. subtilis ccdA* is cotranscribed with two downstream genes, *yneI* and *yneJ*, but neither of the downstream

genes contribute to sporulation or cytochrome *c* maturation (20). *ccdA1* appears to be part of a large gene cluster containing genes predicted to be involved in multiple cellular processes. Of note is the presence immediately downstream of *ccdA1* of an ortholog of *yneN*, a membrane-bound protein with a thioredoxin-like domain that may interact with *ccdA* in *B. subtilis* (25). To determine the structure of a putative *ccdA1* operon, linkage analysis was performed by RT-PCR using 34F2 RNA and sets of primers flanking the intergenic regions of each potential gene pair. As shown in Fig. S1 in the supplemental material, amplicons were obtained for each intergenic region between BAS1644 and BAS1649, indicating that transcription of these genes is linked and constitutes an operon. The genes flanking either end of the BAS1644-9 operon are transcribed in the opposite direction and are not part of the operon. These findings are consistent with a recent global transcriptome analysis that identified *ccdA1* as part of a six-gene operon (40). Linkage analysis of *ccdA2* indicates that it is unlinked to either of the surrounding genes transcribed in the same orientation. The lack of upstream linkage is unsurprising due to the large intergenic region and predicted transcriptional terminator between BAS3456 and *ccdA2*. A linkage between *ccdA2* and BAS3454, a *yneJ*-like gene, might have been expected given the similarities to the operon organization in *B. subtilis* (*ccdA-yneI-yneJ*) (20, 27), but the lack of a *yneI*-like gene between *ccdA2* and the *yneJ*-like BAS3454 indicates genomic dissimilarities between the two species.

**Characterization of *ccdA* deletion strains.** To investigate the role of *ccdA1* and *ccdA2* in virulence gene expression, cytochrome *c* maturation, and sporulation, we created markerless deletion strains in the *B. anthracis* 34F2 background. Strain AW-A027 is missing *ccdA1*, strain AW-A028 is missing *ccdA2*, and strain AW-A029 is missing both *ccdA1* and *ccdA2*. A growth curve in LB of the double-deletion strain is shown in Fig. 1A. Loss of *ccdA1* and *ccdA2* singly (data not shown) or loss of both in the double deletion did not result in significant growth defects.

*B. anthracis* virulence gene expression was measured by  $\beta$ -galactosidase assays using transcriptional fusions of either the *atxA*

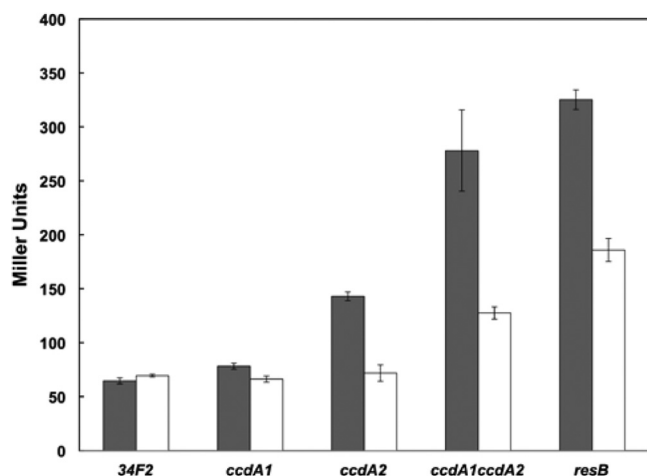


FIG 2 Early *atxA* expression in *ccdA* mutant strains.  $\beta$ -Galactosidase activity of parental and mutant strains carrying the *atxA-lacZ* reporter grown in LB supplemented with kanamycin at 37°C. Filled bars represent *atxA* expression at 3 h postinoculation, and empty bars represent *atxA* expression at 4 h postinoculation.

(41) or *pagA* (5) promoter to an *lacZ* reporter. As shown in Fig. 1B, loss of both *ccdA1* and *ccdA2* resulted in increased transcription of *atxA* during exponential phase and increased transcription of *pagA* during early stationary phase, similar to the phenotype of a cytochrome *c* maturation or a *cccA cccB* knockout mutant, as observed previously (10). As *atxA* overexpression in exponential phase was shown to be the cause of increased *pagA* transcription, we focused more carefully on early *atxA* expression. Figure 2 shows the early *atxA* expression profile of multiple strains. As expected of the parental 34F2 strain, *atxA* expression was low at 3 h and remains unchanged at 4 h. In contrast, the *resB* deletion strain, in which cytochrome *c* production is completely abolished, showed a more than 5-fold increase in *atxA* expression at 3 h that decreased significantly at 4 h, consistent with previous findings (10). The *atxA* transcription profile in the *ccdA1 ccdA2* double-deletion strain was similar to that of the *resB* deletion strain, though the expression level was slightly lower. The *ccdA1* deletion strain resembled the parental 34F2 strain with no increased *atxA* expression. The *ccdA2* deletion strain had increased *atxA* expression relative to that of 34F2 and the *ccdA1* deletion strain but lower than that of the double-deletion strain. These results suggested that loss of both *ccdA1* and *ccdA2* is required for strongly increased virulence gene expression and that there is some degree of complementarity between CcdA1 and CcdA2.

Next, the cytochrome *c* oxidase activity of the mutants was assayed by TMPD staining. TMPD is an artificial electron donor that interacts specifically with cytochrome *c* oxidase (cytochrome *caa*<sub>3</sub>) and is oxidized to a blue-colored product that stains colonies in the presence of active cytochrome *c* in the membrane. Parental and mutant strains were grown on NSMP agar plates, stained with TMPD, and scored for the appearance of blue color. As shown in Table 2, the parental 34F2 and the *ccdA1* deletion strains were stained using TMPD, indicating active cytochrome *c* oxidase. In contrast, the *ccdA2* and *ccdA1 ccdA2* deletion strains were not stained using TMPD, indicating a lack of active cytochrome *c* oxidase. These findings are consistent with the *atxA* transcription results, suggesting that loss of *ccdA2* results in loss of cytochrome *c*, while loss of *ccdA1* has little effect.

*ccdA* is also involved in spore formation in *B. subtilis* in an activity distinct from its role in cytochrome *c* maturation (20). To test the effect of loss of *ccdA1* and *ccdA2* in sporulation of *B. anthracis*, parental and mutant strains were grown on SM. Sporulation efficiency was monitored by both microscopic examination and liquid sporulation assays. As shown in Table 2, the sporulation efficiency of the *ccdA1* deletion strain was similar to that of the parental 34F2 strain, while sporulation of the *ccdA2* and *ccdA1 ccdA2* deletion strains was significantly reduced. Sporulation was also tested using a different sporulation-inducing medium, NSMP, and sporulation efficiency was similar to the results obtained using SM (data not shown). These results suggest that loss of *ccdA2* results in decreased sporulation, while loss of *ccdA1* has no observable effect on sporulation.

***ccdA2* is expressed at higher levels than *ccdA1*.** Our initial characterization of the *ccdA* deletion strains suggests that *ccdA2* is responsible for all observed phenotypes, while loss of *ccdA1* has no measurable effect. This may be due to differential activities of the two protein products, i.e., CcdA1 does not function in cytochrome *c* maturation and sporulation pathways. Alternatively, the results could be due to different levels of gene expression, i.e., *ccdA1* is expressed at much lower levels than *ccdA2* such that loss of CcdA1 can easily be compensated for by the presence of abundant CcdA2. We performed quantitative RT-PCR to measure *ccdA1* and *ccdA2* mRNA levels in the parental 34F2 strain. Assays were performed in triplicate and analyzed by the comparative  $C_T$  ( $2^{-\Delta\Delta C_T}$ ) method relative to the *gyrB* control. Under conditions used in the previous phenotypic characterization assays, *ccdA2* was expressed at levels 14.2 ( $\pm 0.91$ )-fold higher than *ccdA1*. The higher levels of *ccdA2* expression can explain the lack of effect in the *ccdA1* single deletion, as the loss of the smaller amount of CcdA1 can easily be compensated by the much more abundant CcdA2, assuming that protein concentrations are consistent with mRNA amounts. This may also explain the increase in *atxA* expression in the *ccdA1 ccdA2* deletion strain relative to that in the *ccdA2* deletion strain, as the small amount of CcdA1 production lost in the double mutant further reduces *c*<sub>550</sub> and *c*<sub>551</sub> levels and increases *atxA* transcription.

***ccdA1* expression is reduced in sporulation-inducing medium.** The promoter-containing fragments upstream of the *ccdA1* and *ccdA2* operons were cloned into the pTCV-lac vector to monitor the temporal pattern of gene expression by  $\beta$ -galactosidase analysis. Growth and the temporal pattern of *ccdA1* and *ccdA2* expression were examined in rich medium (LB) and in NSMP. As

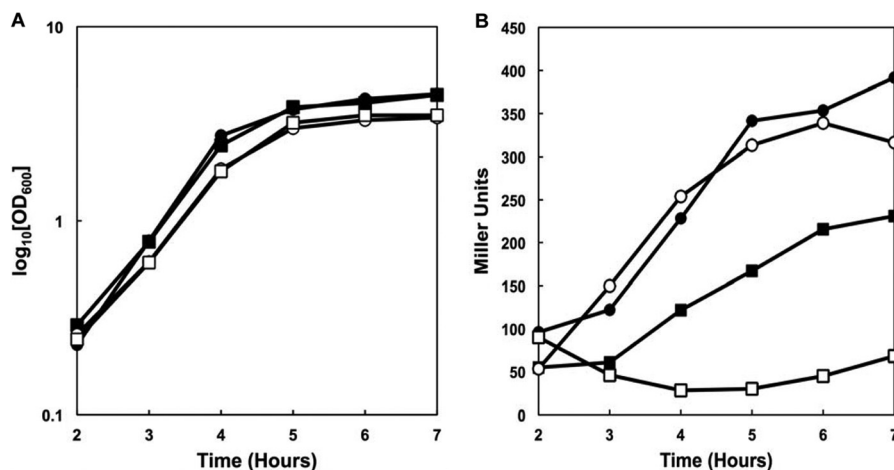
TABLE 2 Cytochrome *c* oxidase activity and sporulation efficiency in strains of *B. anthracis*

Plasmid	TMPD staining <sup>a</sup>				Sporulation efficiency <sup>b</sup>			
	34F2	<i>ccdA1</i>	<i>ccdA2</i>	<i>ccdA1 ccdA2</i>	34F2	<i>ccdA1</i>	<i>ccdA2</i>	<i>ccdA1 ccdA2</i>
pAW285 (Empty)	+	+	—	—	53	55	21	23
pAW304 ( <i>ccdA1</i> )	+	+	+	+	52	51	19	20
pAW305 ( <i>ccdA2</i> )	+	+	+	+	50	53	56	53
pAW306 ( <i>B. subtilis ccdA</i> )	+	+	+	+	ND	55	57	53

<sup>a</sup> +, TMPD staining detected; —, no TMPD staining detected.

<sup>b</sup> Sporulation values represent the percentages of spores relative to the total number of viable cells. Results are the averages from three independent assays. Standard errors of the means were less than 10% of the means in all experiments. ND, not determined.





**FIG 3** Gene expression profile of *ccdA1* and *ccdA2*. (A) Cell growth of the parental 34F2 strain carrying *ccdA1*(pAW355) and *ccdA2*(pAW350) reporter plasmids grown in the medium indicated supplemented with kanamycin at 37°C. (B) β-Galactosidase activity of the parental 34F2 strain carrying *ccdA1*(pAW355) and *ccdA2*(pAW350) reporter plasmids grown in the medium indicated supplemented with kanamycin at 37°C. For both panels: ■, *ccdA1-lacZ* in LB; ●, *ccdA2-lacZ* in LB; □, *ccdA1-lacZ* in NSMP; ○, *ccdA2-lacZ* in NSMP.

shown in Fig. 3A, strains grew more slowly in NSMP than in LB. Consistent with previous quantitative RT-PCR results, expression of *ccdA1* was lower than that of *ccdA2* when grown in LB (Fig. 3B). Expression of *ccdA2* was not significantly altered when grown in NSMP compared to that in LB. In contrast, expression of *ccdA1* was strongly reduced to near background levels when grown in NSMP. Similarly, low *ccdA1* expression levels were observed when cells were grown in SM, another commonly used sporulation-inducing medium (data not shown). These data show that expression of *ccdA1* is strongly reduced under sporulation-inducing conditions, while expression of *ccdA2* is unchanged.

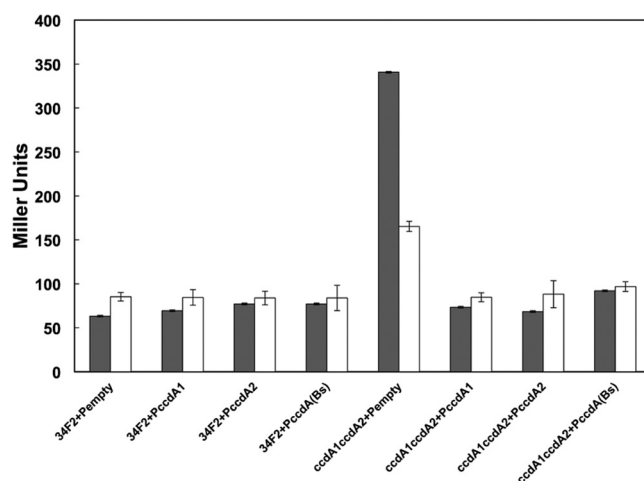
**CcdA2 is active in cytochrome *c* maturation and sporulation, while CcdA1 is active only in cytochrome *c* maturation.** Our earlier analysis of the *ccdA1* and *ccdA2* single-deletion strains was hampered by substantial differences in expression. To eliminate the differences in expression and more directly probe the activities of CcdA1 and CcdA2, we took a complementation approach. *ccdA1*, *ccdA2*, and *B. subtilis ccdA* were cloned into the expression plasmid pAW285. This plasmid places the gene of interest under the control of the *B. subtilis xylA* promoter, which is constitutively active in the absence of added xylose in *B. anthracis* (data not shown), thereby eliminating differences in transcription from the endogenous promoters.

*atxA* gene expression was monitored by β-galactosidase analysis in the parental 34F2 strain and the *ccdA1 ccdA2* double-deletion strain carrying various plasmids (Fig. 4). The addition of the empty pAW285 or any of the CcdA-expressing plasmids did not significantly alter *atxA* expression in the 34F2 strain. The *ccdA1 ccdA2* strain (AW-A029) carrying empty plasmid overexpressed *atxA* in a pattern similar to that of our previous data, suggesting the empty plasmid does not affect expression. Addition of plasmids carrying *ccdA1*, *ccdA2*, or *B. subtilis ccdA*, when expressed at similar levels, can complement the *atxA* overexpression phenotype associated with loss of CcdA activity.

Cytochrome *c* maturation in the parental 34F2 and *ccdA* deletion strains carrying various plasmids was assayed by TMPD oxi-

dase staining (Table 2). The addition of empty or *ccdA*-expressing plasmids did not alter cytochrome *c* oxidase activity in the parental 34F2 strain. The *ccdA2* and *ccdA1 ccdA2* strains transformed with empty plasmid were deficient in TMPD staining, indicating that the empty plasmid does not alter cytochrome *c* maturation. Consistent with the *atxA* expression data, the addition of plasmids carrying *ccdA1*, *ccdA2*, or *ccdA* of *B. subtilis* restored TMPD staining in all deficient strains.

Sporulation efficiency assays were also performed in the complementation strains. As shown in Table 2, the addition of *ccdA2* or *B. subtilis ccdA* returned sporulation to parental strain levels in the *ccdA2* and *ccdA1 ccdA2* deletion strains. Surprisingly, the addition of *ccdA1* was unable to complement the sporulation defect in the *ccdA2* and *ccdA1 ccdA2* deletion strains. The addition of



**FIG 4** Early *atxA* expression in parental and *ccdA1 ccdA2* mutant strains carrying *ccdA* expression plasmids. β-Galactosidase activity of parental and mutant strains carrying the *atxA-lacZ* reporter grown in LB supplemented with kanamycin and chloramphenicol at 37°C. Filled bars represent *atxA* expression at 3 h postinoculation, and empty bars represent *atxA* expression at 4 h postinoculation.

**TABLE 3** Cytochrome *c* oxidase activity and sporulation efficiency in strains of *B. subtilis*

Plasmid	TMPD staining <sup>a</sup>		Sporulation efficiency <sup>b</sup>	
	JH642	<i>ccdA</i>	JH642	<i>ccdA</i>
pAW285 (Empty)	+	—	34	2
pAW304 ( <i>ccdA1</i> )	+	—	37	3
pAW305 ( <i>ccdA2</i> )	+	—	34	34
pAW306 ( <i>B. subtilis ccdA</i> )	+	+	35	34

<sup>a</sup> +, TMPD staining detected; —, no TMPD staining detected.<sup>b</sup> Sporulation values represent percentages of spores relative to the total number of viable cells. Results are the averages from three independent assays. Standard errors of the means were less than 10% of the means in all experiments.

xlyose to the cultures, which increases expression levels almost 5-fold, did not alter sporulation in the presence of the *ccdA1* complementation plasmid (data not shown), demonstrating that the inability to complement is not the result of expression levels. While *ccdA2* and *B. subtilis ccdA* can complement all phenotypes associated with loss of CcdA activity, *ccdA1* was incapable of complementing sporulation deficiency.

**Neither *ccdA1* nor *ccdA2* can fully complement the loss of *ccdA* in *B. subtilis*.** Previous observations indicate that *ccdA* of *B. subtilis* provided in *trans* can fully complement the phenotypes associated with loss of *ccdA1* and *ccdA2* in *B. anthracis*. We investigated the converse: whether *ccdA1* and *ccdA2* can complement the loss of *ccdA* in *B. subtilis*. A markerless deletion strain missing *ccdA* was constructed in the JH642 strain of *B. subtilis*. This strain, AW-S002, was defective in cytochrome *c* oxidase activity, as measured by loss of TMPD oxidase activity, and had reduced sporulation efficiency relative to parental JH642. AW-S002 was transformed with the same complementation plasmids previously used in *B. anthracis*. Unlike in *B. anthracis*, neither *ccdA1* nor *ccdA2* could restore TMPD oxidase activity in the *ccdA* deletion strain (Table 3). In contrast, *ccdA* of *B. subtilis* could restore TMPD oxidase activity in the *ccdA* deletion strain. When sporulation efficiency was measured in the same strains, *ccdA2* and *B. subtilis ccdA* could restore sporulation to parental strain levels, while the addition of *ccdA1* did not affect sporulation, observations similar to what was seen in *B. anthracis*. The same complementation plasmid stocks used for the *B. subtilis* experiments were again independently transformed into both *B. anthracis* and *B. subtilis*, and these strains showed the same phenotypes previously reported, excluding plasmid irregularities, as an explanation of our observations. Neither *ccdA1* nor *ccdA2* could complement cytochrome *c* oxidase deficiency, while only *ccdA2* could complement sporulation deficiency, indicating that the activities of *B. anthracis* CcdA1 and CcdA2 are dissimilar when expressed in *B. subtilis* and *B. anthracis*.

## DISCUSSION

Membrane-bound thiol-disulfide oxidoreductases contribute to several processes important to metabolism and virulence in *B. anthracis*. There is conservation among components between *B. anthracis* and the well-characterized model organism *B. subtilis*. One significant difference lies in the fact that, unlike *B. subtilis*, which carries one *ccdA*, *B. anthracis* carries two copies of *ccdA*. In *B. anthracis*, CcdA2 functions in cytochrome *c* maturation, *atxA* regulation, and sporulation. In contrast, CcdA1 functions in cyto-

chrome *c* maturation and *atxA* regulation but has no activity in efficient sporulation. The actual activities of the two proteins are partially masked by the significant differences in expression levels.

The presence of two distinct *ccdA* genes is a feature found throughout the pathogenic *B. cereus* group. Comparison of genomic sequences deposited to GenBank reveals a *ccdA1 ccdA2* pair in all screened genomes of the pathogenic *B. cereus* group, including *B. anthracis*, *B. cereus*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis*. In contrast, most other nonpathogenic environmental bacilli, including *B. subtilis*, *Bacillus pumilus*, and *Bacillus halodurans*, carry only one *ccdA*, which is more similar to *ccdA1*. Interestingly, some other bacilli, including *Geobacillus* spp. and *Bacillus licheniformis*, carry multiple *ccdA1*-like genes. The presence of a distinct *ccdA2*-like gene appears to be common in the pathogenic bacillus group, suggesting the genetic events that generated two distinct *ccdA* genes occurred following separation of the *B. cereus* group from the other bacilli. The potential contribution of *ccdA2*-like genes to pathogenicity of these organisms may merit further investigation.

The operon organization of *ccdA1* and *ccdA2* is distinct from that of *B. subtilis ccdA*. *ccdA1* is the fourth gene in a six-gene operon. The purpose of the linkage of these genes is unclear, as most of the genes have no known function, and predictions of activity based on similarity of conserved domains reveals little commonality. A gene of interest in the operon is the fifth gene, BAS1648, just downstream of *ccdA1*, which is predicted to encode a protein with 59.4% amino acid similarity to *B. subtilis* YneN. YneN is one of the 8 membrane-bound thiol-disulfide oxidoreductase proteins in *B. subtilis*, a group which also includes CcdA, ResA, StoA, BdbA, BdbB, BdbC, and BdbD (14, 25, 42, 43). Unlike CcdA, which is a polytopic membrane protein, YneN possesses a single transmembrane segment (24). YneN is unique among this group in that it has no known function in *B. subtilis* and clearly is not involved in either cytochrome *c* maturation or sporulation (24, 25). The proximity of the genes suggests that CcdA1 and the YneN-like protein might be a functional pair, with CcdA1 serving as the electron donor to the YneN-like protein, as has been previously suggested (27).

The gene expression profiles of *ccdA1* and *ccdA2* are consistent with their observed activities. When grown on rich medium, *ccdA1* and *ccdA2* are expressed robustly. The relevance of significantly higher expression of *ccdA2* than *ccdA1* in the parental 34F2 strain grown in rich medium is unclear but could perhaps be related to reduced need for *ccdA1* given its reduced spectrum of cellular activity. When grown on sporulation-inducing media, *ccdA2* expression was unchanged, but *ccdA1* expression dropped to near background levels. As CcdA1 is not needed in sporulation, lack of expression under sporulation-inducing conditions could be expected. Experimental induction of artificially high transcription of either *ccdA1* or *ccdA2* using our complementation plasmids does not alter the phenotypes of the parental strain or the complementation strains without induction, suggesting that increased production of CcdA does not strongly affect *B. anthracis* physiology.

When the *B. anthracis ccdA* complementation plasmids were placed in a *B. subtilis ccdA* deletion strain, a different pattern of activity emerged than that seen in *B. anthracis*. We found that neither CcdA1 nor CcdA2 was active in cytochrome *c* maturation, which is odd given the similarity in the target thiol-disulfide oxi-

doreductase in cytochrome *c* maturation, ResA, in both organisms. CcdA2 could restore sporulation, while CcdA1 could not, identical to the situation in *B. anthracis*. This, too, is unexpected in light of our previous findings given the low degree of similarity between the CcdA sporulation target thiol-disulfide oxidoreductase, StoA, and any other predicted thiol-disulfide oxidoreductase in the *B. anthracis* genome. We cannot rule out the possibility that our *in trans* complementation approach may not accurately reflect the activities of native CcdA, but the internal consistency of these experiments does highlight functional differences. These observations suggest that specificity of thiol-disulfide oxidoreductases cannot easily be predicted and is dependent upon factors beyond the conservation of the active cysteine residues.

Thiol-disulfide oxidoreductases have been implicated in the pathogenesis of several organisms, including *Escherichia coli* (44, 45), *Vibrio cholerae* (46), and *Haemophilus influenzae* (47), among many others (reviewed in reference 48). The effect of the loss of *ccdA1* and *ccdA2* on virulence of *B. anthracis* is unclear. The virulence gene dysregulation upon loss of cytochrome *c* activity is unique: increased *atxA* only during exponential phase, which leads to increased toxin expression in stationary phase though a mechanism still under investigation. This effect, however, is seen only under *in vitro* conditions that do not induce strong toxin production (10). It was recently reported that manipulation of the *atxA* promoter to overexpress AtxA did not alter virulence in a murine model of infection (49). While this experiment did not directly replicate the unique *atxA* expression profile found in the loss of *ccdA1* and *ccdA2*, it does raise questions about the relevance of AtxA overexpression. It is also unclear as to whether loss of *c*-type cytochromes at the cell surface has any effect on virulence of *B. anthracis*. Beyond the connection between cytochrome *c* maturation and virulence regulation through AtxA, the association between CcdA and spore formation provides a link to *B. anthracis* pathogenicity. Spores of *B. anthracis* are the infectious particle and are capable of persisting outside the host under adverse environmental conditions. Germination of spores to vegetative cells allows production of virulence factors, but the vegetative cells are incapable of initiating infection (1, 50); thus, the process of sporulation is essential to perpetuate the infectious cycle of *B. anthracis*. Factors that alter or reduce sporulation, such as loss of CcdA1 and CcdA2, disrupt the pathogenic life cycle of *B. anthracis* and can significantly reduce the infectious success of *B. anthracis*.

While these observations provide a unique view of the function and specificity of these duplicate *ccdA* genes, they also highlight the gaps in our knowledge of thiol-disulfide oxidoreductases. Thiol-disulfide oxidoreductases are essential to correct assembly of surface-exposed proteins that are important to many processes in *B. subtilis*, including sporulation (20, 21), competence (43), cytochrome maturation (18), and lantibiotic production (51). It is certainly possible that thiol-disulfide oxidoreductases could be involved in other as-yet-undefined processes in Gram-positive bacteria. Further, thiol-disulfide oxidoreductases are an interesting drug target, given their roles in the pathogenesis of many organisms and evidence suggesting that inhibitors can block viral infection (52). Additional work is required to define the relationships of CcdA1 and CcdA2 and other thiol-disulfide oxidoreductases to their cognate targets in order to define their activities in bacterial physiology and virulence.

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